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(71) Applicant *(for all designated States except US)*: SEBO GMBH [DE/DE]; Oppertsweg 6, D-64711 Erbach (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BOOS, Karl-Siegfried [DE/DE]; Elisabethstrasse 4, D-82131 Gauting (DE). SEIDEL, Dietrich [DE/DE]; Pschorrstrasse 21, D-82340 Feldafing (DE). SELLERGREN, Börje [SE/DE]; Lennigstrasse 7, D-55118 Mainz (DE). WIESCHEMEYER, Jürgen [DE/DE]; Kaiser-Wilhelm-Ring 44, D-55118 Mainz (DE).

(74) Agents: WEICKMANN, H. et al.; Kopernikusstrasse 9, D-81679 München (DE).

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(57) Abstract

The present invention refers to a method for selective adsorption of cholesterol from aqueous fluids and cholesterol-imprinted adsorber copolymers suitable therefor.

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Imprinted copolymers for selective adsorption of cholesterol

Description

The present invention refers to a method for selective adsorption of cholesterol from aqueous fluids and cholesterol-imprinted adsorber copolymers suitable therefor.

There is overwhelming evidence that hypercholesterolemia is the major risk factor for the early development of atherosclerosis in man and thus the leading cause of coronary heart and peripheral atherosclerotic disease (1). On the basis of results from various intervention studies it is well established that drastic lowering of blood cholesterol concentration is followed by a reduction of clinical events, of morbidity and total mortality.

Cholesterol homeostasis is regulated by the amount of cholesterol absorbed from the diet, by hepatic cholesterol synthesis, metabolism and by hepatic sterol excretion. With the introduction of a new family of substances (HMG-CoA reductase inhibitors) it became possible to increase hepatic LDL receptor activity and by this cholesterol excretion. Specific inhibitors of cholesterol absorption from the diet are not available so far and attempts to achieve cholesterol reduction by such a strategy were less impressive (2,3). Because in clinical practice it is often necessary to efficiently interfere with both absorption and excretion of cholesterol to achieve the recommended blood cholesterol concentrations, one approach would be to develop cholesterol selective adsorbents that are biocompatible, easily accessible and clinically efficient.

One way of imparting molecular recognition properties to a material is by the way of molecular imprinting (4-6). A few approaches to imprint cholesterol have been described to date (7-9). Whitcombe et al. showed

that cholesterolselective adorbents can be prepared by a covalent molecular imprinting strategy. The system made use of an easily cleavable carbonate ester linkage between a phenol monomer and cholesterol during polymerization. After polymerization and removal of the template rebinding was driven by hydrogen bonding between the hydroxyl group of cholesterol and the polymeric phenols (7). The rebinding of cholesterol to these materials was evaluated in hexane showing a fairly homogenous population of binding sites with a dissociation constant of 0.59 mM and a binding capacity of 114 μmol/g. These materials were only evaluated in hexane and no data is given for the rebinding in water. It is also possible to obtain enhanced binding of cholesterol using approaches based on noncovalent self-assembly of the monomer and cholesterol (8,9). Recently, Asanuma et al. described the recognition properties exhibited by polymers prepared by crosslinking of a low molecular weight host, β-cyclodextrin, with diisocyanates in presence of cholesterol (9). The materials were capable of rebinding cholesterol also in aqueous media.

Strong and selective rebinding in water depends on the hydrophobic effect and requires a large Van der Waals contact area between cholesterol and the host (10,11). This can be provided by cyclodextrins (*vide supra*) but, as we describe in this application, may also be provided by imprinting of cholesterol using apolar association between the monomers and the template.

Molecular imprinting based on entropically driven association between the functional monomers and the template was previously described in the imprinting of 2,4-dichlorobenzoacetic acid (12). In this case recognition was more pronounced for materials prepared using polar protic solvents and at higher temperatures, all in agreement with expectations on entropically driven associations. Furthermore, a number of examples have shown that compounds can rebind to imprinted sites with a specific hydrophobic driving force (13,14).

In this application we have synthesized polymerizable derivatives of cholesterol and bile acids (Scheme 1) to be used as apolar monomers in the imprinting of highly crosslinked methacrylates with cholesterol. The polymers have been prepared under conditions favoring apolar intermolecular interactions (Scheme 2) and have been evaluated in the chromatographic mode or in intestinal mimicking fluids in the batch mode.

Steroidfunctional polymers imprinted with cholesterol exhibit enhanced affinity and capacity for cholesterol in intestinal-mimicking media. The adsorptive capacity seems to be unrelated to the specific surface area of the materials but are instead due to binding sites induced by the presence of steroid units in the polymer backbone as well as the presence of cholesterol during formation of the adsorbent. The templating effect of cholesterol probably involves apolar interactions with the apolar parts of the monomers during polymerization. This may result in hydrophobic binding pockets capable of accomodating cholesterol in the subsequent rebinding experiment. Cholesterol itself or other steroids offer the best binding sites for cholesterol. The crystal structure of cholesterolmonohydrate features layers of close-packed cholesterol molecules with a large apolar contact area and a polar sheet of hydrogenbonded hydroxyl groups and water molecules (7). The cholesterol monomer may interact with cholesterol in a similar fashion. Accessory monomers prepared from other steroids are also suited for cholesterol binding materials, e.g. methacryloyl derivatives of ergosterol, stigmasterol, testosterone or β-sitosterol. Alternatively another site of coupling of the functional monomer to cholesterol may lead to stronger interactions.

An alternative recognition mechanism involves the existence of cholesterol clusters. This may seem even more likely considering that a porogen, ethanol, is preferably used as a recrystallization solvent for cholesterol. Moreover the cholesterol monomer may stabilize such clusters. More detailed information regarding the imprinting mechanism may be achieved

from spectroscopic characterizations of the solution complexes present prior to polymerization. The adsorptive capacity exhibited by the cholesterol imprinted resins as well as their cost and ease of preparation are promising for a future therapeutic use of imprinted polymers for preventing diet-cholesterol related diseases. The adsorbents may also be useful in other applications relying on strong and selective binding of stereoids in aqueous media.

Thus, a first aspect of the present invention is a method for selective adsorption of cholesterol from aqueous fluids comprising: (a) providing a cholesterol-imprinted adsorber material comprising steroid groups capable of binding to cholesterol and (b) contacting an aqueous fluid containing cholesterol with the adsorber material under conditions which allow adsorption of cholesterol to the adsorber material.

The aqueous fluid is preferably a biological fluid which may contain proteins and/or lipids such as triglycerides, phospholipids etc. More preferably, the aqueous fluid is a body fluid, e.g. plasma or gastro-intestinal fluid.

The adsorber material is preferably a cholesterol-imprinted organic polymer comprising steroid groups. The term "cholesterol-imprinted" means that the polymer has been synthesized in the presence of cholesterol. The polymer is synthesized from at least two monomers (i) and (ii) wherein (i) is a vinyl group-containing monomer which preferably carries a group which is negatively charged under physiological conditions, e.g. a carboxy or sulfonic acid group. The monomer (ii) is a vinyl group-containing monomer having a steroid group capable of binding to cholesterol. The monomer (ii) may be synthesized by reacting steroid compounds, e.g. via their OH-groups with vinyl group-containing monomers, e.g. by forming an ester bond.

The monomer (i) may be a single species of molecules or a mixture of several species. Preferred examples are acrylic and/or methacrylic acid.

Preferred examples for the monomer (ii) are acryloyl and/or methacryloyl derivatives of steroid group-containing compounds such as cholesterol or bile acids, particularly cholic acid or desoxycholic acid, and esters, e.g. methylesters thereof.

The cholesterol-imprinted adsorber material preferably has a porous and cross-linked structure. Therefore the polymer preferably further contains a cross-linking monomer (iii) which is compatible with monomers (i) and (ii) and which contains at least two polymerizable groups, e.g. vinyl groups. Preferred examples for the cross-linking monomers (iii) are ethylene glycol dimethacrylate and/or ethylene glycol diacrylate.

A further aspect of the present invention is a cholesterol-imprinted adsorber material comprising steroid groups capable of binding to cholesterol. This adsorber material is preferable produced by a method comprising the steps: (a) polymerizing at least two monomers (i) and (ii) wherein (i) is a vinyl group-containing monomer having a negatively charged group and (ii) is a vinyl group-containing monomer having a steroid group capable of binding to cholesterol in the presence of cholesterol and a porogen and (b) removing the cholesterol. For the polymerization preferably a further cross-linking monomer (iii) as defined above is present.

The porogen is an organic solvent like, e.g. dichlormethane, toluene, acetonitrile or an alcohol such as ethanol. Preferred are polar organic solvents like, e.g. acetonitrile and in particular ethanol. The polymerization reaction is preferably carried out as homogeneous reaction using the porogen as a solvent.

The removal of cholesterol may be carried out by extractive methods, which leaves distinct cavities within the polymer network, the topochemistry of which is complementary in size, shape and functionality to the cholesterol

template. These cavities (or imprinted sites) allow the specific rebinding of cholesterol molecules and thereby molecular recognition.

Still a further aspect of the present invention is a method for selective adsorption of cholesterol from aqueous fluids comprising: (a) providing an adsorber material comprising a tomatin capable of binding to cholesterol and (b) contacting an aqueous fluid containing cholesterol with the adsorber material under conditions which allow adsorption of cholesterol to the adsorber material.

The methods for selective adsorption of cholesterol from aqueous fluids as described above are suitable for the qualitative or quantitative determination of cholesterol in a sample, e.g. a biological sample. The determination of cholesterol may be carried out by adding labelled cholesterol analogs to the sample and then determining the amount of label bound to the adsorber which is inversely correlated to the amount of cholesterol in the sample.

A preferred use of the methods for selective adsorption of cholesterol from aqueous fluids is however the manufacture of a medicament against cholesterol-related disorders, e.g. hypercholesterolemia, or obesity. Thus a further aspect of the present invention is a pharmaceutical composition comprising the cholesterol-imprinted adsorber material as defined above optionally together with pharmaceutically acceptable diluents, carriers and adjuvants. The pharmaceutical composition is preferably administered orally. However, also extra-corporeal treatments , e.g. the extra-corporeal treatment of body fluids such as blood plasma, are encompassed by the present invention. The pharmaceutical composition may be used in combination with other pharmaceutical agents which modulate the enterohepatic metabolism and/or circulation such as pankreas lipase inhibitors.

The cholesterol-imprinted adsorber material according to the invention is preferably a physiologically compatible polymer, which is substantially insoluble under physiological conditions. Thus, any treatment, e.g. the oral administration is to a large extent free of undesired side effects. Hence, exemplary daily doses of the adsorber material according to the invention are from 1-25 g, in particular from 5-15 g.

Moreover, the present invention shall be explained by the following figures and examples.

Figure description

Scheme 1

Formulae of different steroid monomers

Scheme 2

General schema for the synthesis of cholesterol-imprinted polymers

Scheme 3

Formulae of different steroid components

Figure 1

Scanning electron micrographs of polymers P11 (a), P12 (b), P13 (c) and P14 (d) photographed at 3000 x magnification.

Figure 2

Capacity factors for cholesterol (Cho), ergosterol (Erg) and testosterone (Tes) on columns packed with the adsorbents shown in Table 1 in a mobile phase consisting of (a) methanol/water 95 (v/v) and (b) methanol/water: 90/10 (v/v). Conditions otherwise as described in the experimental section.

Figure 3

Capacity factors for the various steroids versus the amount of water in the mobile phase for the adsorbents subjected to extraction in soxhlet apparatus and drying. (a) P11 and P12, (b) P13 and P14 (log k'). The hydrophobicity of the steroids estimated by computer program is: Cho: 9.8, Sti: 10.2 Sit: 10.7, Erg: 9.3, Tes: 3.5.

Figure 4

Adsorption isotherms (a-c) of cholesterol in intestine-mimicking medium (5 ml) at pH 7.5 using the adsorbents (30 mg) described in the experimental section and in Table 1. The samples were stirred in a circularly shaking bath at 37°C for 24 h and the amount of cholesterol thereafter determined enzymatically. The experiment was repeated twice for each adsorbent.

Experimental section

1. Material and methods

1.1 Chemicals

The monomers ethylene glycol dimethacrylate (EDMA) (98%) (Aldrich) and a methacrylic acid (MAA) (99%) (Aldrich) were purified as previously described and stored in a refrigerator. EDMA was washed with 10% sodium hydroxide, dried with anhydrous sodium sulfate and distilled whereas MAA was purified by distillation. The initator α,α'-azo-bis-(isobutyronitrile) (AIBN)

was obtained from Janssen and purified by recrystallisation from methylenechloride and stored dry and cold.

The HPLC-grade ethanol used in the polymerizations contained 5% isopropanol and 5% methanol and was stored over molecular sieve 4Å (Aldrich). The other solvents used (Merck) were of p.a.-grade and were stored over molecular sieve 4Å and the water collected from a Millipore water purification system.

Cholic acid ($\geq$ 99%), desoxycholic acid ($\geq$ 99%), cholesterol (Cho) (from Lanolin, $\geq$ 99%), testosterone (Tes) ($\geq$ 99%), N,N'-dicyclohexylcarbodiimide (DCC) (~ 99%), 3-glycidyloxypropyl-trimethoxysilane ($\geq$ 97%), α-tomatine (lycopersicin) (~ 99%) and sodium desoxycholate ($\geq$ 99%) were purchased from Fluka and used without further purification. Stigmasterol (Sti) (95%), β-sitosterol (Sit) (50%), ergosterol (Erg) (95%), 4-dimethylaminopyridine (DMAP) (99%), sodium cholate (98%), acetic acid (99,8%), potassium dihydrogenphosphate (99+%), sodium hydrogencarbonate (99%), sodium hydroxide (97+%) and bortrifluoride-ethyletherate (redistilled) came from Aldrich.

The enzyme assay used for the determination of free cholesterol (*Cholesterol 50*®) was from Sigma and was stored at 4°C. The adsorbent Amberlite® XAD2000 was purchased from Sigma whereas LiChrosorb® Si 100, 100 μm, was kindly provided by Dr. K.-F. Krebs (Merck, KGaA, Darmstadt). The C18 modified silicas used in the batch experiments (M5.15, M5.20) were provided by Dipl.-Chem. Frank Großmann (Universität Mainz).

<u>1.2 Equipment and characterization</u>

The temperature during polymerization was regulated using an automated thermostat (Fryka-Therm FT 800). The UV-lamp used in the

photopolymerization was a high-pressure mercury vapor lamp (Philips, HPK 125 W). The polymers were ground in a ballmill (Pulverisette 05.202, Fritsch) and sieved using stainless steel sieves from Retsch (DIN 4188). The polymers were packed in chromatographic columns using an airdriven liquid pump (Haskel DSTV-122). The chromatographic evaluations of the imprinted polymers were done using a Hewlett Packard instrument (HP1050) equipped with a quaternary pump, an auto-sampler, a variable wavelength detector and an HP work station. The pore- and surface area analysis was done by nitrogen sorption using a ASAP 2010 instrument from Micromeritics and the scanning electron micrographs obtained at the University of Mainz pathology department. The polymer density was estimated by weighing an amount of polymer (particle size 25-36 μm) corresponding to 1 ml into graduated NMR-tubes. The swelling was then estimated by equilibrating the polymer in acetonitrile over night followed by tapping until no further change in bed height was observed. The amount of extracted template was determined after two different extraction procedures. In the first, the polymers were stirred in methanol for 2 x 24 h followed by evaporation of the extracts and enzymatic assay for cholesterol. In the second, the polymers were subjected to consecutive soxhlet extractions for 5 h in methanol and methylene chloride, respectively, and then drying of the polymer at 60°C over night. The extracts were evaporated and redissolved in $CDCl_3$ containing benzene as international standard. The cholesterol content was then estimated by comparing the ^{1}H-NMR integrals.

The log P values of the steroids were estimated by the incremental method using the software ACD/log P1.13, Toronto, Canada, available on the internet.

1.3. Synthesis of imprinted polymers

The polymers were synthesized following the general imprinting protocol shown in Scheme 2 and the monomer compositions given in Table 1. A typical procedure would be as follows: EDMA (5.88 g, 30 mmol), MAA (0.52 g, 6 mmol) and the steroid monomer (3 mmol) were dissolved in ethanol or dichloromethane (9 ml). For the imprinted polymers cholesterol (0.58 g, 1.5 mmol) was added and the solution gently heated. Thereafter the initiator AIBN (50 mg) was added. The clear solution was transferred to a thick-walled glass polymerization tube, cooled on ice, degassed by sparging with nitrogen gas for 10 minutes and then sealed. In the thermochemically initiated polymerizations, the tube was immersed into a water bath maintained at 60°C (ethanol) or 38°C (dichlormethane). In the photochemically initiated polymerizations the tubes were allowed to equilibrate at 10°C for 10 minutes. Then the tube was irridiated using a high pressure Hg lamp rotating the tube once 180°C within the first 15 minutes. The polymerization time was in all cases 16 hours. Following polymerization the polymer monolyth was crushed in a mortar and then ground in wetted state by means of a mechanical ball mill followed by sieving. The procedure was optimized so as to obtain the maximum yield of the required size fraction, 25-35 μm.

1.4 Synthesis of tomatin-silica-adsorbents

Synthesis of epoxy-silica

To a suspension of LiChrosorb Si 200, 10 μm, (5g) in toluene (200 ml), 3-glycidyloxypropyl-trimethoxysilane (18 g, 76 mmol) was added dropwise. After heating the suspension to reflux for 5 h, the gel was filtered off and washed with acetone, methanol, acetone and diethylether followed by drying under vacuum. Yield: 5.91 g dry solid. Anal: Found: C 7.74%, H 1.64%.

Synthesis of tomatin-silica

The synthesis was carried out analogously to the method described in (15). To a solution of α-tomatin (lycopersicin) (200 mg, 0.19 mmol) in 20 ml 1,4-dioxane, dry epoxy-silica (1 g) was added. After addition of bortrifluoride-ethyletherate (1 ml) the reaction was left for 48 h at room temperature whereby the flask was shaken three times. The modified silica was filtered and washed with 1,4-dioxane, methanol, methanol/water, methanol, 1,4-dioxane and diethylether. Thereafter it was dried at 80°C under vacuum. Yield: 1.05 g dry solid. Anal: Found: C 10.98%, H 2.13%, N 0.07%.

1.5 Synthesis of steroid monomers

Cholic acid methylester

Cholic acid acid (40 g, 98 mmol) was dissolved in methanol (200 ml) followed by addition of 1.5 ml concentrated HCl and heating of the solution to reflux for 30 minutes. This resulted in a dark yellow colored solution. After leaving the solution at room temperature crystallization started. The solution was left for 48 h at 4-8°C and thereafter filtered cold. This gave 35.8 g of colorless crystals. Yield: 86,1%.

^{1}H-NMR (400 MHz, $CDCl_3$), δ / ppm = 0.64 (s, 3H, 18-H_3), 0.85 (s, 3H, 19-H_3), 0.94 (d, 3H, 21-H_3, $J_{21\text{-}H,20\text{-}H}$ = 6 Hz), 1.33-1.86 (Steroid), 2.16 (m, 2H, 22-H_2), 2.34 (m, 2H, 23-H_2), 3.43 (m, 1H, 3β-H), 3.63 (s, 3H, COOCH_3), 3.80 (s, 1H, 7β-H), 3.92 (s, 12β-H)
^{13}C-NMR (100.6 MHz, $CDCl_3$): δ / ppm = 12.25 (18-C), 17.09 (21-C), 22.23 (19-C), 23.02, 26.09, 27.31, 27.92, 30.09, 30.72, 30.91, 34.45, 34.57, 35.11, 39.27, 41.28, 41.37, 46.20, 46.77, 50.33, 51.29 (COO$\underline{C}H_3$), 68.25 (7-C) 71.68 (3-C), 72.89 (12-C), 174.66 ($\underline{C}OOCH_3$).

EI-MS: m/z (%): 422 (1; M^+), 404 (7; M^+-H_2O), 386 (100; M^+-2 H_2O), 371 (10; 386-CH_3), 368 (37; M^+-3 H_2O), 355 (10; 386-OCH_3), 353 (14; 368-CH_3), 271 (33), 253 (16)
IR (KBr): ν/cm^{-1} = 3401, 2934, 2870, 1738, 1447, 1379, 1171, 1080, 1032, 982
Anal. calc. for $C_{25}H_{42}O_5$ (422.61): C 71.05, H 10.02; found: C 70.89, H 10.14

Desoxycholic acid methylester

Desoxylcholic acid, (50 g, 127 mmol), was dissolved in 200 ml methanol under gentle heating followed by addition of 1.5 ml concentrated HCl and heating of the solution to reflux for 30 minutes. This resulted in a red-brown colored solution. After addition of a small volume of water the solution was left for 48 h at 4-8 °C and thereafter crystals were separated by filtration. This gave 37.9 g colorless crystals. Yield: 73.3%.
^{1}H-NMR(400 MHz, $CDCl_3$): δ / ppm = 0.64 (s, 3H, 18-H_3), 0.87 (s, 3H, 19-H_3), 0.92 (d, 3H, 21-H_3, $J_{21\text{-}H,20\text{-}H}$ = 5.7 Hz), 1.21-1.79 (Steroid), 2.19 (m, 2H, 22-H_2), 2.33 (m, 2H, 23-H_2), 3.56 (m, 1H, 3β-H), 3.62 (s, 3H, $COOC\underline{H}_3$), 3.94 (s, 1H, 12β-H)
^{13}C-NMR(100.6 MHz, $CDCl_3$): δ / ppm = 12.50 (18-C), 17.04 (21-C), 22.92 (19-C), 23.48, 25.93, 27.29, 28.42, 30.18, 30.69, 30.92, 33.38, 33.90, 35.02, 35.06, 35.81, 36.18, 41.87, 46.27, 47.02, 47.98, 51.29 ($COO\underline{C}H_3$), 71.46 (3-C), 72.89 (12-C), 174.55 ($\underline{C}OOCH_3$)
EI-MS: m/z (%): 406 (4; M^+), 388 (59; M^+-H_2O), 370 (100; M^+-2H_2O), 357 (19; 388-OCH_3), 355 (27; 370-CH_3), 273 (72), 255 (60)
IR (KBr): ν/cm^{-1} = 3432, 2938, 2864, 1742, 1449, 1377, 1169, 1044
Anal. calc. for $C_{25}H_{42}O_4$ (406.61): C 73.85, H 10.41; found: C 73.61, H 10.52.

3α-methacryloyl-desoxycholic acid methylester (DCAMe1MAA)

Desoxycholic acid methylester, (6 g, 14.8 mmol), DCC, (3.36 g, 16.3 mmol) and DMAP, (0.21 g, 1.7 mmol), were dissolved in dichloromethane (100 ml). Thereafter MAA, (1.41 g, 16.4 mmol) was added dropwise and the reaction allowed to proceed over night under stirring. The dicyclohexylurea was filtered off and the solution washed with water, 5% acetic acid, 0.5 N sodium bicarbonate and brine (each 60 ml). After drying of the organic phase with anhydrous sodium sulfate the solution was taken down resulting in a white solid. The solid was treated with water, 80 ml, heated to 50°C for 15 minutes and the remaining solid filtered and dried. Yield: 5.93 g (84.4%) of a white amorphous solid.

^{1}H-NMR(400 MHz), $CDCl_3$): δ / ppm = 0.65 (s, 3H, 18-H_3), 0.89 (s, 3H 19-H_3), 0.94 (d, 3H, 21-H_3, $J_{21\text{-H},20\text{-H}}$ = 6.2 Hz), 1.02-1.95 (Steroid), 1.89 (s, 3H, 3-O(CO)C($C\underline{H_3}$)=CH_2), 2.22 (m, 2H, 22-H_2), 2.33 (m, 2H, 23-H_2), 3.63 (s, 3H, $COOC\underline{H_3}$), 3.96 (s, 1H, 12β-H), 4.74 (m, 1H, 3α-H), 5.47, 6.03 (s, 1H, 3-O(CO)C(CH_3)=$C\mathit{H_2}$),

^{13}C-NMR(100.6 MHz, $CDCl_3$): δ / ppm = 12.52 (18-C), 17.12 (21-C), 18.14 (3-O(CO)C($\underline{C}H_3$)=CH_2), 22.93 (19-C), 23.39, 25.80, 26.34, 26.77, 27.22, 28.57, 30.69, 30.82, 31.98, 33.44, 33.50, 33.94, 35.78, 41.69, 46.28, 47.13, 48.10, 51.28 ($COO\underline{C}H_3$), 57.93, 72.93 (3-C), 74.32 (12-C), 124.75 (3-O(CO)C(CH_3)=$\underline{C}H_2$), 136.68 (3-O(CO)$\underline{C}$(CH_3)=CH_2), 166.85 (3-O($\underline{C}$O)C(CH_3)=CH_2), 174.49 ($\underline{C}OOCH_3$)

EI-MS: m/z (%): 474 (1, M^+), 456 (2; M^+-H_2O), 388 (18; M^+-MAA), 370 (49; 388-H_2O), 355 (15; 370-CH_3), 341 (17), 273 (16), 255 (100)

IR (KBr): ν/cm^{-1} = 3551, 2934, 2864, 1739, 1699, 1630, 1452, 1298, 1188, 1044

Anal. calc. for $C_{29}H_{46}O_5$ (474.68): C 73.38, H 9.77; found: C 73.15, H 9.81.

3α,12α-di-methacryloyl-desoxycholic acid methylester (DCAMe2MAA)

Starting with desoxycholic acid methylester, (6.1 g, 15 mmol), DCC, (6.82 g, 33 mmol), DMAP, (0.4 g, 3.3 mmol) and MAA (2.92 g, 34 mmol) the synthesis took place as described for DCAMe1MAA. The white residue obtained after evaporation was recrystallized from 25 ml acetone.
Yield: 7.13 g (87.6%) of colorless crystals.
^{1}H-NMR(400 MHz, $CDCl_3$): δ / ppm = 0.69 (s, 3H, 18-H_3), 0.94 (s, 3H 19-H_3), 0.97 (d, 3H, 21-H_3, $J_{21\text{-}H,20\text{-}H}$ = 6 Hz), 1.18-2.01 (Steroid), 1.93 (s, 3H, 3-O(CO)C(C$\underline{H_3}$)=CH_2), 1.99 (s, 3H, 12-O(CO)C(C$\underline{H_3}$)=CH_2), 2.24 (m, 2H, 22-H_2), 2.41 (m, 2H, 23-H_2), 3.67 (s, 3H, COOC$\underline{H_3}$), 4.78 (m, 1H, 3α-H), 4.93 (s, 1H, 12α-H), 5.53, 6.08 (s, 1H, 3-O(CO)C(CH_3)=C$\underline{H_2}$),
^{13}C-NMR(100.6 MHz, $CDCl_3$): δ / ppm = 12.54 (18-C), 17.15 (21-C), 18.15 (3-O(CO)C($\underline{C}H_3$)=CH_2), 19.81 (12-O(CO)C($\underline{C}H_3$)=CH_2), 22.94 (19-C), 24.47, 25.33, 25.81, 26.25, 26.36, 28.53, 30.70, 30.83, 32.43, 33.52, 33.96, 35.80, 41.71, 46.30, 47.17, 48.13, 51.30 (COO$\underline{C}H_3$), 58.12, 72.98 (12-C), 74.30 (3-C), 124.74 (3-O(CO)C(CH_3)=$\underline{C}H_2$), 136.70 (3-O(CO)$\underline{C}$(CH_3)=CH_2), 166.85 (3-O($\underline{C}$O)C(CH_3)=CH_2), 174.48 ($\underline{C}OOCH_3$)
EI-MS: m/z (%): 456 (15; M^+-MAA), 438 (1; 456-H_2O), 370 (96; M^+-2 MAA), 355 (17; 370-CH_3), 341 (12), 255 (100)
IR (KBr): ν/cm^{-1} = 3061, 2936, 2859, 1709, 1649, 1452, 1350, 1175, 1017,
Anal. calc. for $C_{33}H_{50}O_6$ (542.75): C 73.03, H 9.29; found: C72.91, H 9.43.

3-α-methacryloyl-cholic acid methylester (CAMe1MAA)

Starting with cholic acid methylester (7.2 g, 17 mmol), DCC, (3.87 g, 18.7 mmol), MAA, (1.61 g, 18.7 mmol) and DMAP, (0.24 g, 1.9 mmol) the synthesis took place as described for DCAMe1MAA. The solid obtained after evaporation was recrystallized from 40 ml ethylacetate.
Yield: 7.70 g (92%) of white crystals.

^{1}H-NMR(400 MHz), $CDCl_3$): δ / ppm = 0.65 (s, 3H, 18-H_3), 0.86 (s, 3H 19-H_3), 0.96 (d, 3H, 21-H_3, $J_{21\text{-}H,20\text{-}H}$ = 5.5 Hz), 1.02-2.08 (Steroid), 2.02 (s, 3H, 3-O(CO)C($C\underline{H_3}$) = CH_2), 2.19 (m, 2H 22-H_2), 2.35 (m, 23-H_2), 3.63 (s, 3H, COOC$\underline{H_3}$), 3.96 (s, 1H, 12α-H),4.74 (m, 1H, 3α-H), 5.51 (d, 1H, 3-O(CO)C(CH_3) = C$\underline{H_2}$, $J_{vinyl\text{-}H}$ = 16 Hz),

^{13}C-NMR(100.6 MHz, $CDCl_3$): δ / ppm = 12.25 (18-C), 17.09 (21-C), 18.07 (3-O(CO)C($\underline{C}H_3$) = CH_2), 22.24 (19-C), 23.01, 24.46, 26.13, 27.30, 27.93, 30.14, 30.88, 32.39, 34.46, 34.55, 35.08, 39.28, 41.28, 41.41, 46.21, 46.77, 51.28 (COO$\underline{C}H_3$), 57.94, 68.24 (7-C), 71.66 (3-C), 72.86 (12-C), 124.69 (3-O(CO)C(CH_3) = $\underline{C}H_2$), 136.71 (3-O(CO)$\underline{C}$(CH_3) = CH_2), 166.84 (3-O-($\underline{C}$O)C(CH_3) = CH_2, 174.56 ($\underline{C}$OOCH$_3$)

EI-MS: m/z (%): 454 (1; M^+-2 H_2O), 404 (1; M^+-MAA), 386 (7; 404-H_2O), 368 (6; 386-H_2O), 271 (6), 253 (7)

IR (KBr): ν/cm^{-1} = 3509, 3067, 2934, 2857, 1739, 1697, 1628, 1452, 1348, 1215, 1080, 910

Anal. calc. for $C_{29}H_{46}O_6$ (490.68): C 70.99, H 9.45; found: C 70.64, H 9.42.

3α,7α-di-methacryloyl-cholic acid methylester (CAMe2MAA)

Cholic acid methylester, (6.4 g, 15 mmol), DCC, (6.86 g, 33 mmol), MAA, (2.92 g, 34 mmol) and DMAP, (0.4 g, 3.3 mmol), were dissolved in dichlormethane (100 ml). The synthesis was carried out as described for DCAMe1MAA. The white amorphous solid obtained after evaporation of dichloromethane was recrystallized from 50 ml acetone/water: 85/15 (v/v). Yield: 5.87 g (69.4%) of white crystals.

^{1}H-NMR(400 MHz, $CDCl_3$): δ / ppm = 0.65 (s, 3H, 18-H_3), 0.86 (s, 3H 19-H_3), 0.92 (d, 3H, 21-H_3, $J_{21\text{-}H,20\text{-}H}$ = 3.5 Hz), 1.12-2.12 (Steroid), 1.89 (s, 3H, 3-O(CO)C(C$\underline{H_3}$) = CH_2), 1.94 (s, 3H, 7-O(CO)C$\underline{H_3}$) = CH_2), 2.24 (m, 2H, 22-H_2), 2.31 (m, 2H, 23-H_2), 3.61 (s, 3H, COOC$\underline{H_3}$), 3.95 (s, 1H, 12α-H), 4.66 (m, 3α-H), 5.00 (s, 1H, 7α-H), 5.48 (d, 2H, 3-O(CO)C(CH_3) = C$\underline{H_2}$, 7-

$O(CO)C(CH_3)=C\underline{H}_2$, $J_{vinyl-H}$ = 16 Hz), 6.02 (d, 2H, 3-$O(CO)C(CH_3)=C\underline{H}_2$, 7-$O(CO)C(CH_3)=C\underline{H}_2$, $J_{vinyl-H}$ = 16 Hz)

^{13}C-NMR(100.6 MHz, $CDCl_3$): δ / ppm = 12.57 (18-C), 17.08 (21-C), 18.07 (3-$O(CO)C(\underline{C}H_3)=CH_2$), 19.72 (7-$O(CO)C(\underline{C}H_3)=CH_2$), 22.24 (19-C), 23.01, 24.49, 25.02, 26.13, 26.37, 27.27, 30.49, 30.64, 30.80, 32.29, 34.49, 35.04, 39.19, 41.05, 46.28, 46.92, 51.25 ($COO\underline{C}H_3$), 57.46, 67.98 (7-C), 72.75 (3-C), 74.47 (12-C), 124.61 (3-$O(CO)C(CH_3)=\underline{C}H_2$), 125.14 (7-$O(CO)C(CH_3)=\underline{C}H_2$), 136.48 (7-$O(CO)\underline{C}(CH_3)=CH_2$), 136.68 (3-$O(CO)\underline{C}(CH_3)=CH_2$), 166.80 (3-$O(\underline{C}O)C(CH_3)=CH_2$), 166.67 (3-$O(\underline{C}O)C(CH_3)=CH_2$), 174.56 ($\underline{C}OOCH_3$)

EI-MS: m/z (%): 292 (6), 211 (15), 167 (9), 98 (22), 86 (24), 69 (100)

IR (KBr): ν/cm^{-1} = 3542, 2936, 2863, 1739, 1628, 1448, 1331, 1175, 1074, 1016, 912

Anal. calc. for $C_{33}H_{50}O_7$ (558.75): C 70.94, H 9.02; found: C 70.76, H 9.04.

3α,7α,12α-tri-methacryloyl-cholic acid methylester (CAMe3MAA)

Cholic acid methylester, (6.8 g, 16 mmol), DCC, (10.95 g, 53 mmol), MAA, (4.56 g, 53 mmol) and DMAP, (0.7 g, 5.7 mmol) were dissolved in dichloromethane (100 ml) and stirred over night. The synthesis was carried out as described for DCAMe1MAA. The organic phase was washed with water, 5% acetic acid, saturated sodium bicarbonate solution, water and brine and then dried over anhydrous sodium sulfate. The white amorphous solid obtained after evaporation was recrystallized from 30 ml acetone.

Yield: 6.42 g (76.6%) of a white amorphous solid.

^{1}H-NMR(400 MHz, $CDCl_3$): δ / ppm = 0.64 (s, 3H, 18-H_3), 0.90 (s, 3H 19-H_3), 0.92 (d, 3H, 21-H_3, $J_{21-H,20-H}$ = 8.0 Hz), 1.12-2.27 (Steroid), 1.85 (s, 3H, 3-$O(CO)C(C\underline{H}_3)=CH_2$), 1.91 (s, 3H, 12-$O(CO)C(C\underline{H}_3)=CH_2$), 1.94 (s, 3H, 7-$O(CO)C(C\underline{H}_3)=CH_2$), 2.27 (m, 2H, 22-$H_2$), 2.31 (m, 2H, 23-$H_2$), 3.61 (s, 3H, $COOC\underline{H}_3$), 4.61 (m, 1H, 3α-H), 4.95 (s, 7α-H), 5.09 (s, 1H, 12α-H), 5.48 (d, 3H, 3-$O(CO)C(CH_3)=C\underline{H}_2$, 7-$O(CO)C(CH_3)=C\underline{H}_2$, 12-

$O(CO)C(CH_3)=C\underline{H}_2$, $J_{vinyl\text{-}H}$ = 15 Hz), 6.04 (t, 3H, 3-$O(CO)C(CH_3)=C\underline{H}_2$), 7-$O(CO)C(CH_3)=C\underline{H}_2$, 12-$O(CO)C(CH_3)=C\underline{H}_2$, $J_{vinyl\text{-}H}$ = 16 Hz)

^{13}C-NMR(100.6 MHz, $CDCl_3$): δ / ppm = 12.30 (18-C), 17.16 (21-C), 18.05 (3-$O(CO)C(\underline{C}H_3)=CH_2$), 18.27 (12-$O(CO)C(\underline{C}H_3)=CH_2$), 19.79 (7-$O(CO)C(\underline{C}H_3)=CH_2$), 22.34 (19-C), 22.77, 24.46, 25.04, 25.32, 26.23, 26.45, 27.04, 30.55, 30.81, 32.40, 34.50, 34. 74, 38.21, 41.94, 47.02, 49.31, 51.29, ($COO\underline{C}H_3$), 58.01, 70.90 (7-C), 72.40 (3-C), 73.88 (12-C), 125.16 (3-$O(CO)C(CH_3)=\underline{C}H_2$, 7-$O(CO)C(CH_3)=\underline{C}H_2$, 12-$O(CO)C(CH_3)=\underline{C}H_2$), 136.68 (3-$O(CO)\underline{C}(CH_3)=CH_2$, 7-$O(CO)\underline{C}(CH_3)=CH_2$, 12-$O(CO)\underline{C}(CH_3)=CH_2$), 166.71 (3-$O(\underline{C}O)C(CH_3)=CH_2$, 7-$O(\underline{C}O)C(CH_3)=CH_2$, 12-$O(\underline{C}O)C(CH_3)=CH_2$), 174.56 ($\underline{C}OOCH_3$)

EI-MS: m/z (%): 292 (6), 211 (15), 167 (9), 98 (22), 86 (24), 69 (100)

IR (KBr): ν/cm^{-1} = 2934, 2858, 1739, 1699, 1628, 1452, 1333, 1194, 1015, 910

Anal. calc. for $C_{33}H_{50}O_7$ (626.83): C 70.90, H 8.68; found: C 70.72, H 8.59.

3α-methacryloyl-cholesterol (ChoMAA)

Cholesterol, (5 g, 13 mmol), DCC, (3.5 g, 17 mmol), MAA (1.45 g, 17 mmol) and DMAP, (0.2 g, 1.7 mmol), were dissolved in dichloromethane (100 ml) and stirred for 48 h at room temperature. After filtering off the dicycohexylurea the organic phase was washed with water, 5% acetic acid, saturated sodium bicarbonate solution, water and brine and then dried over anhydrous sodium sulfate. The colorless oil obtained after evaporation was recrystallized from 20 ml ethylacetate.

Yield: 5.56 g (94%) of white crystals.

^{1}HNMR(400 MHz, $CDCl_3$): δ / ppm = 0.63 (s, 3H, 18-H_3), 0.83 (d, 3H 27-H_3, $J_{27\text{-}H,25\text{-}H}$ = 1.7 Hz), 0.88 (d, 3H, 21-H_3, $J_{26\text{-}H,25\text{-}H}$ = 6.5 Hz), 0.99 (s, 3H, 19-H_3), 1.04-2.32 (Steroid), 1.89 (s, 3H, 3-$O(CO)C)C\underline{H}_3)=CH_2$), 4.61 (m, 1H, 3$\alpha$-H), 5.33 (m, 1H, 6-H), 5.48 (s, 1H, 3-$O(CO)C(CH_3)=C\underline{H}_2$), 6.03 (s, 1H, 3-$O(CO)C(CH_3)=C\underline{H}_2$),

^{13}C-NMR(100.6 MHz, $CDCl_3$): δ / ppm = 11.62 (18-C), 18.13 (21-C), 18.51 (3-O(CO)C($\underline{C}H_3$)=CH_2), 19.14 (21-C), 20.84 (19-C), 22.36, 22.67, 23.64, 24.08, 27.57, 28.03, 27.04, 31.67, 31.74, 35.59, 36.80, 37.91, 39.38, 39.54, 42.11, 49.82, 55.93, 56.49, 56.57, 74.01, 122.44 (6-C), 124.74 (3-O(CO)C(CH_3)=$\underline{C}H_2$), 136.66 (3-O(CO)$\underline{C}$(CH_3)=CH_2), 139.49 (5-C), 166.67 (-O($\underline{C}$O)C(CH_3)=CH_2)

EI-MS: m/z (%): 375 (18), 369 (24; M^+-MAA), 354 (66; 369-CH_3), 129 (89), 117 (100), 91 (81)

IR (KBr): v/cm^{-1} = 2936, 2854, 1721, 1649, 1466, 1375, 1294, 1169, 1013, 935

Anal. calc. for $C_{31}H_{52}O_2$ (454.73): C 81.52, H 11.48; found: C 81.51, H 11.41

1.6 Chromatographic evaluation of the imprinted polymers

The polymer particles (size 25-36 μm) were sedimented in 100 ml methanol followed by sedimentation twice in methanol/water: 80/20 (v/v), the second time accompanied by sonication. The particles were then slurry packed into HPLC columns (125x4 mm, Merck) in 80% aqueous methanol at pressures of 200-300 bar.

The columns were equilibrated in methanol until a stable baseline was achieved, usually within 30-45 min. The flow rate was 1 ml/min, the UV detector wavelength 220 nm (cholesterol, stigmasterol and β-sitosterol), 271 nm (ergosterol), 241 nm (testosteron) or 254 nm (acetone) and the chromatography run at room temperature with duplicate injections unless otherwise stated. The retention, k', was calculated as $k' = (t-t_o)/t_o$ where t_o is the elution time of the void marker, acetone which normally eluted as a sharp peak with a maximum plate number, N, of approximately 10000/m. A volume of 10 μl of stock solutions of the steroids (2 mg/10 ml) in the mobile phase were injected separately.

1.7 Batch rebinding experiments in intestinal-mimicking medium

Preparation of intestinal-mimicking medium (A)

To 125 ml of a 0.2 M potassium dihydrogen phosphate solution and 95 ml of a 0.2 M sodium hydroxide solution was added 200 ml water. Then 24.5 g sodium desoxycholate (NaDC) and 16.5 g sodium cholate (NaC) was added and dissolved by stirring. This gave the solution a light yellow color. The pH was adjusted to 7.5 ± 0.1 with 0.2 M sodium hydroxide and water added to a final volume of 500 ml. After sparging with nitrogen for 30 min the solution was stored in darkness at room temperature.

Preparation of cholesterol standard solution (B)

To 500 ml of A was added cholesterol (901.7 mg) and the solution treated for 3 hours at 50°C under sonication. The solution was then sparged with nitrogen for 30 min and stored in darkness at room temperature.

Rebinding experiment

The dry adsorbents (30 mg) were weighed into 20 ml glass vials and 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 or 5.0 ml of the cholesterol standard solution (B) added followed by addition of solution A to a final volume of 5 ml. The samples were then stirred in a circulary shaking bath at 37°C for 24 h. After sedimentation of the adsorbents 30 μl of the supernatant was transferred to an enzyme reagent solution (3 ml) (Sigma kit no 352-50) and left for 5 min at 37°C for completion of the reaction. The absorbance at 500 nm was then measured. The amount cholesterol adsorbed was calculated by subtraction using a calibration curve obtained from the same experiment leaving out the adsorbent. The experiment was repeated at least twice for each adsorbent.

2. Results and discussion

2.1 Functional monomer design

Cholesterol is a hydrophobic steroid (estimated log P_{ow} = 9.8) with a relatively planar structure and with one polar hydroxyl substituent in the A ring (Scheme 3). As in the biological recognition elements efficient recognition of hydrophobic molecules is achieved using hosts with lipophilic binding sites offering large apolar contact area towards the guest (10,11). As shown in a previous work, molecular imprinting can produce sites capable of discriminating cholesterol from sitosterin (Scheme 3), a structurally similar analog, in hexane (7). Addition of only small amounts of more polar solvents completely supressed the selective binding. In water, no recognition elements so far reported are capable of this level of recognition. In this work our objective was to explore the steroid backbone as a building block in the templating of recognition sites for cholesterol. Bile acids belong to one class of easily accessible steroids that possess amphiphilic properties with a polar face that can be further derivatized (2,10). They have therefore been used in the construction of macrocycles for molecular recognition. Furthermore they have, as their monomethacrylate derivatives, been copolymerized with polar methacrylate monomers to form random bile acid containing copolymers (16). In light of these facts we considered them a suitable first choice in the cholesterol templating work. We thus synthesized a number of mono-, di- and trimethacrylate-substituted bile acid methyl esters (Scheme 1). Due to the low reactivity of the 7α and 12α OH groups, acylation using methacryloyl anhydride or methcryloyl chloride failed. In this case only DMAP catalyzed esterification gave the desired products in good yield. Parallel to the synthesis of the bile acid derivatives O-methacryloyl-cholesterol was also synthesized. In view of the crystal structure of cholesterol (17) it may provide the most complementary surface for binding cholesterol.

2.2 Polymer synthesis and physical characterization

Polymers imprinted with cholesterol were synthesized following a previously described procedure with some modifications. The polymers were all prepared by free radical terpolymerization of a mixture of methacrylic acid (MAA), the crosslinking monomer ethylene glycol dimethacrylate (EDMA) and the steroid monomer as described in the experimental section and in Table 1. MAA was used in order to obtain hydrogen bonding to the cholesterol hydroxyl group and to provide the polymer with negative charges for repelling of the bile acids in the intestine. By the use of polar protic solvents it was anticipated that apolar association of the steroid monomers and cholesterol would be favored. Using an excess of functional monomer intermolecular assemblies of the type shown in Scheme 2 would provide the hydrophobic binding sites necessary for strong and selective rebinding of cholesterol. Obviously this relies on a preference for intermolecular association of the type A-B at the expense of A-A and B-B, respectively. After polymerization the polymers were freed from cholesterol by washing with methanol at room temperature. This was compared with a soxhlet extraction in methanol and dichloromethane. The room temperature methanol wash resulted in a splitting yield of 39-57% whereas after the soxhlet extraction in methanol the yield was nearly quantitative (Table 2). The polymers were characterized by nitrogen sorption analysis, SEM and swelling measurements. This revealed small differences between the imprinted and nonimprinted polymers and larger differences between the polymers prepared using the different porogens. First of all, previous characterization of linear copolymers of MAA and 3O-methacrylolyl cholic acid has indicated a high yield of polymerization and a random incorporation of the monomers. Furthermore we could not identify peaks that could be assigned to unreacted monomer in the NMR-spectra of the soxhlet extracts. The swelling and porosity of the polymers were in agreement with previous observations (18). Thus polymers prepared using ethanol as porogen can be characterized as macroporous with relatively low swelling whereas

polymers prepared using dichlormethane as porogen are gellike with low porosity in the dry state and a significant swellability. Typically the nitrogen adsorption isotherms as well as the SEMs of the ethanol polymers (Figure 1) indicated a significant amount of pores with a diameter larger than 1000 Å. Furthermore, these pores appeared more frequently in the imprinted compared to the nonimprinted polymers. The former polymers showed furthermore a larger swelling in acetonitrile and a higher dry density than the latter polymers. Previously small differences between imprinted and blank polymers have been observed. These have been explained by considering the crosslinking function of the template. After its removal the imprinted polymer will be less densely crosslinked than the blank non-imprinted polymer and will thus swell more in good solvating solvents (19).

2.3 Chromatographic characterization

In Figure 2 the calculated capacity factors for the different steroids injected on the different columns packed with cholesterol-imprinted and nonimprinted polymers are plotted. The first evaluation was done using polymers that had not been subjected to extractions using the soxhlet extractor. Thus the template was extracted on line in the chromatographic mode. Considering the particle size of the packings the number of theoretical plates were in most cases acceptable in between 200-1000 indicating a fair column efficiency. For lower plate numbers it was verified that no void had been created at the column inlet. First of all it was seen that the retention of the hydrophobic steroids cholesterol and ergosterol increased strongly with the water content in the mobile phase whereas the less hydrophobic testosterone was relatively weakly retained and did not respond to the addition of water. The linear dependance of log k' on the water content (Figure 3B) further supports that the retention is mainly controlled by the hydrophobic effect. Relatively strong retention was seen on the polymers prepared using dichloromethane as porogen (P3, P4, P9, P10). This may be related to a more efficient solvation of the hydrophobic

monomers leaving them more exposed after removal of the porogen. Comparing the retention on the polymers P7 and P8 with P9 and P10 it is seen that the cholic acid monomer promotes an almost doubling of the capacity factors for the hydrophobic steroids. For the dichloromethane polymers, however, no difference between the retention on the imprinted and the blank polymers (P7 and P8 or P9 and P10, respectively) was observed. When dichloromethane was used as mobile phase the retention was weak and similar on both polymers. A different picture emerged when comparing the polymers prepared using ethanol as porogen. A more than twice larger capacity factor was observed for cholesterol on the imprinted (P1, P5, P11) compared to the blank polymers (P2, P6 and P12) prepared using the cholic acid monomers. Furthermore using the mobile phase consisting of 5% water in methanol the former polymers preferentially retained cholesterol over the similar steroid ergosterol, an effect that disappeared at higher aqueous contents (Figure 2). These observations support a templating mechanism driven by apolar association of monomers and template prior to polymerization. Using dichloromethane as porogen, such association is unlikely due to efficient solvation of the apolar parts of the monomers and the template. In this case, however, hydrogen bonding between MAA and the hydroxy group of the template can be expected to occur. As has been observed by other workers, this stabilization is not strong enough to cause an observable templating effect of cholesterol (7). The type of cholic acid monomer only seems to have a small influence on the templating effect. However the polymers prepared using methacryloyl-cholesterol (P13, P14) instead of the cholic acid monomers exhibited the strongest retention of the hydrophobic steroids among the tested polymers. Although the imprinted polymer retained cholesterol more strongly than the blank polymer the difference was smaller compared to the difference seen for the cholic acid polymers. Nevertheless these polymers clearly exhibited the strongest retention of cholesterol among materials synthesized. However, even stronger retentions were observed on a commercial reversed phase C18 column under the same conditions. On a column with similar

dimensions using methanol/water: 95/5 (v/v) as mobile phase, the capacity factor for cholesterol was 18. However as will be discussed in the next section the affinity of the phases for cholesterol in presence of high concentrations of bile salts is a better indicator for the materials' usefullness under physiological conditions. Chromatographic evaluation of P11-P14 were also done after the soxhlet extraction treatment. As seen in Figure 3 the retention of the steroids was similar as before extraction whereas the selectivity of the imprinted polymers was lower. Porous MIPs are known to be less thermally stable than nonporous ones (18). Thus it is possible that the extraction procedure and the subsequent drying of the polymers at 60°C has denatured the binding sites to some extent.

2.4 Cholesterol adsorption in intestine-mimicking medium

In order to investigate the materials' performance in vivo, the adsorption of cholesterol by the different adsorbents in a medium that closely mimics the intestinal fluid was tested. The solubility of cholesterol in the medium needed to be sufficient to cover a concentration interval that would lead to saturation of the adsorbent binding sites. A mixture of the sodium salts of cholic and desoxycholic acid was found to best satisfy these criteria. An ionic strength and pH corresponding to that found in intestine was adjusted by addition of sodium hydroxide and potassium dihydrogen phosphate. This led to a medium that dissolved 1,8 mg/ml of cholesterol.

The batch experiment was carried out at 37°C under circular stirring i.e. under conditions that would mimic the in vivo conditions. After 24 h the adsorbents were allowed to settle and the supernatant analyzed enzymatically for cholesterol. The assay used is based on coupled reactions catalyzed by cholesterol-oxidase and peroxidase resulting in the formation of a dye in amounts proportional to the amount of cholesterol. The dynamic range for the assay is 15.5 mM with expected values between 1.4-8.2 mM, according to the manufacturers' specifications. A careful control of the

temperature and time of reaction and a regularly repeated calibration are important. The amount adsorbed was calculated by substracting the amount found in the supernatant after adsorption from the amount present before addition of adsorbent. These were then plotted against the equilibrium concentration of free cholesterol in the supernatant (Figure 4). The amount adsorbed by the C18 modified silica adsorbents was similar and increased linearly throughout the concentration range studied, i.e. no saturation was observed. In spite of the higher specific surface area of the hydrophobic Amberlite XAD2000 adsorbent this adsorbed significantly less cholesterol than the C18 silicas. This may be due to the more hydrophobic character of this adsorber leading to agglomeration preventing a fine suspension to form. Silicagel modified with tomatine, a complex glycosylated steroid known to complex cholesterol in aqueous media (20), clearly adsorbed more cholesterol than the precursor epoxy-silica gel or naked silica gel. This was particularly apparent in the low concentration range below 2 mM.

The cholesterol imprinted copolymers exhibited the highest affinity for cholesterol among the tested adsorbents. Particularly striking is the strong adsorption exhibited by P13, the imprinted adsorbent prepared using O-methacryloylcholesterol as functional monomer, which also retained cholesterol most strongly among the imprinted adsorbents in chromatography. Also worth noting are the differences in the binding exhibited by the imprinted and the nonimprinted blank polymers. Physiologically relevant concentrations of cholesterol are expected to lie below 1 mM. In this concentration range P13 together with the tomatin-adsorbent adsorbed cholesterol most strongly of all tested adsorbents. At a cholesterol concentration of 1 mM P13 adsorbs about 45 μmol/g (ca. 17 mg/g) adsorbent whereas the nonimprinted polymer P14 adsorbed ca. 33 μmol/g (ca. 13 mg/g).

Table 1

Preparation of adsorbents prepared for the cholesterol binding experiments

Polymer	Steroid monomer	Template	Porogen	Procedure
P1	DCAMe1MAA	Cholesterol	EtOH	60°C
P2	DCAMe1MAA	-	EtOH	60°C
P3	DCAMe1MAA	Cholesteol	CH_2Cl_2	38°C
P4	DCAMe1MAA	-	CH_2Cl_2	38°C
P5	DCAMe2MAA	Cholesterol	EtOH	60°C
P6	DCAMe2MAA	-	EtOH	60°C
P7	-	Cholesterol	CH_2Cl_2	photo.10° C
P8	-	-	CH_2Cl_2	photo.10° C
P9	DCAMe2MAA	Cholesterol	CH_2Cl_2	photo.10° C
P10	DCAMe2MAA	-	CH_2Cl_2	photo.10° C
P11	CAMe2MAA	Cholesterol	EtOH	60°C
P12	CAMe2MAA	-	EtOH	60°C
P13	ChoMAA	Cholesterol	EtOH	60°C
P14	ChoMAA	-	EtOH	60°C

The polmyers were prepared as described in the experimental section using EDMA (5.88 g, 30 mmol) as crosslinking monomer, the steroid monomer (3 mmol) and MAA (0.52 g, 6 mmol). The templated polymers were prepared in the presence of cholesterol (0.58 g, 1.5 mmol). The porogen was either ethanol or dichloromethane (9 ml) and the polymers were polymerized either by thermochemical initiation at 60°C or photochemically at 10°C.

Table 2

Characterization of adsorbents prepared for the cholesterol binding experiments

Polymer	Surface area[a] (m^2/g)	Pore volume[b] (cm^3/g)	Pore diameter[c] (nm)	Micropore volume[d]	Swelling[e] (ml/ml)	Density[f] (g/ml)
P11	184	0.79	172	0.080	1.32	0.42
P12	198	0.92	186	0.088	1.20	0.34
P13	273	1.05	154	0.12	1.24	0.32
P14	291	1.03	142	0.13	1.16	0.32

Physical characterization of the 25-36 μm particle size fraction. Prior to characterizaton the polymers were extracted in a soxhlet apparatus in methanol and dichloromethane and dried at 60°C as described in the experimental section. The yield of cholesterol after a wash of the polymers in methanol at room temperature was for P11: 57% and for P13: 53% whereas after the soxhlet extraction cholesterol was quantitatively extracted from P13. In the nitrogen adsorption measurements the polymers were outgassed at 40°C for 12 h.

(a) BET surface area using a 40 point pressure table

(b) Total pore volume of pores less than 2600 Å

(c) Average pore diameter (BJH)

(d) DR method micropore volume

(e) Swelling in acetonitrile

(f) Weight of 1 ml of dry polymer (25-36 μm)

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Claims

1. A method for selective adsorption of cholesterol from aqueous fluids comprising:
   (a) providing a cholesterol-imprinted adsorber material comprising steroid groups capable of binding to cholesterol and
   (b) contacting an aqueous fluid containing cholesterol with the adsorber material under conditions which allow adsorption of cholesterol to the adsorber material.

2. The method of claim 1 wherein said aqueous fluid is a body fluid.

3. The method of claim 2 wherein said body fluid is gastro-intestinal fluid.

4. The method of any one of claims 1-3 wherein the adsorber material is an organic copolymer comprising steroid groups.

5. The method of claim 4 wherein the copolymer is synthesized from at least two monomers (i) and (ii) wherein
   (i) is a vinyl group-containing monomer having a negatively charged group and
   (ii) is a vinyl group-containing monomer having a steroid group capable of binding to cholesterol.

6. The method of claim 5 wherein the monomer (i) is selected from acrylic acid and/or methacrylic acid.

7. The method of claim 5 wherein the monomer (ii) is selected from acryloyl and/or methacryloyl derivatives of steroid group-containing compounds.

8. The method of claim 7 wherein the steroid group-containing compounds are selected from cholesterol and bile acids, particularly cholic acid or desoxycholic acid, and esters thereof.

9. The method of any one of claims 5-8 wherein the copolymer further contains a monomer (iii) which is a cross-linking monomer.

10. The method of claim 9 wherein the cross-linking monomer (iii) is selected from ethylene glycol dimethacrylate and/or ethylene glycol diacrylate.

11. A method for selective adsorption of cholesterol from aqueous fluids comprising:
   (a) providing an adsorber material comprising a tomatin capable of binding to cholesterol and
   (b) contacting an aqueous fluid containing cholesterol with the adsorber material under conditions which allow adsorption of cholesterol to the adsorber material.

12. Use of the method of any one of the previous claims for the qualitative or quantitative determination of cholesterol.

13. Use of the method of any one of the previous claims for the manufacture of a medicament against hypercholesterolemia or obesity.

14. A cholesterol-imprinted adsorber material comprising steroid groups capable of binding to cholesterol.

15. A pharmaceutical composition comprising the cholesterol-imprinted adsorber material of claim 14.

16. The use of the cholesterol-imprinted adsorber material for the manufacture of a medicament against hypercholesterolemia.

17. The use of claim 16 in combination with other pharmaceutical agents which modulate the enterohepatic metabolism and/or circulation.

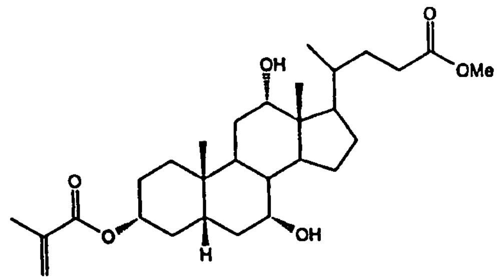
18. The use of claim 17 in combination with pankreas lipase inhibitors.

19. A method for producing a cholesterol-imprinted adsorber material comprising steroid groups capable of binding to cholesterol comprising the steps.
    (a) polymerizing at least two monomers (i) and (ii) wherein
        (i) is a vinyl group-containing monomer having a negatively charged group and
        (ii) is a vinyl group-containing monomer having a steroid group capable of binding to cholesterol in the presence of cholesterol and a porogen and
    (b) removing the cholesterol.

20. The method of claim 19 or 20 wherein the porogen is a polar organic solvent, particularly ethanol.

Scheme 1

CAMe1MAA:



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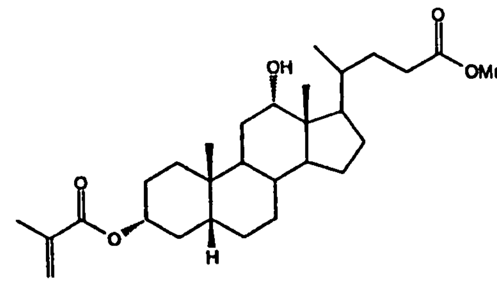

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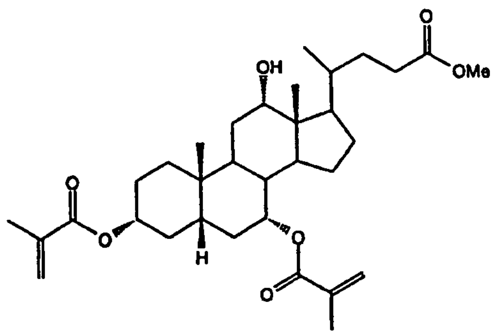

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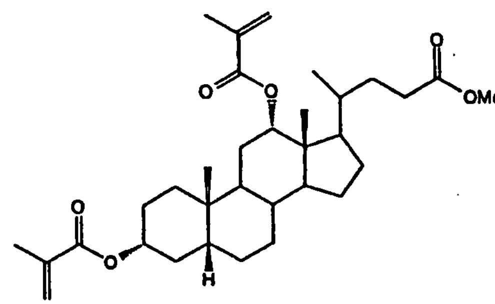

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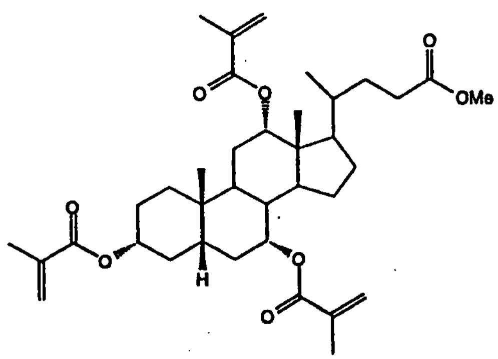
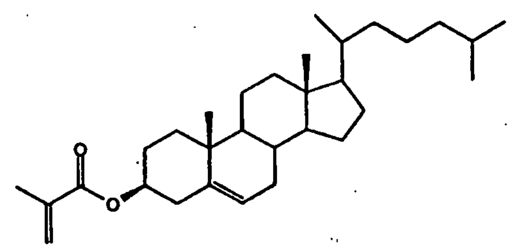


ChoMAA:



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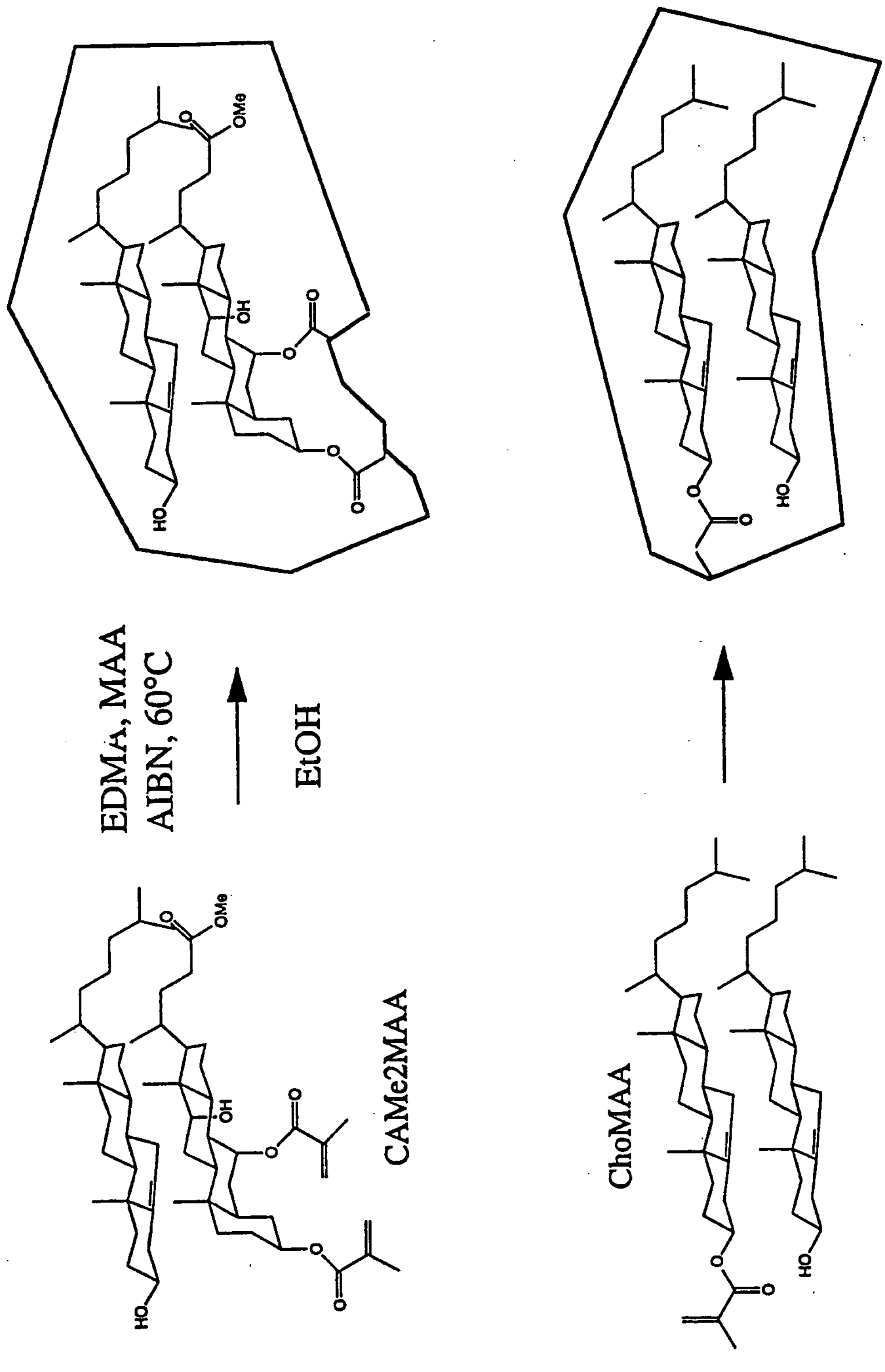
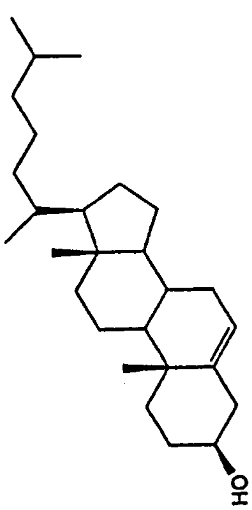


Scheme 3



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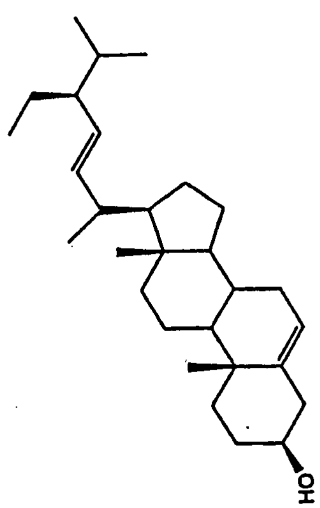

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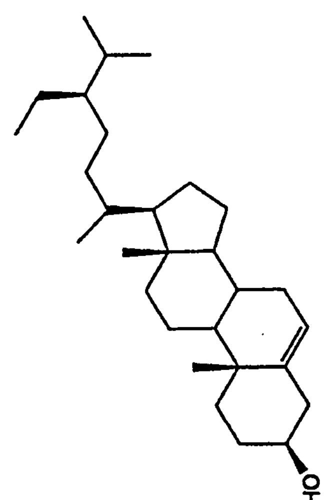

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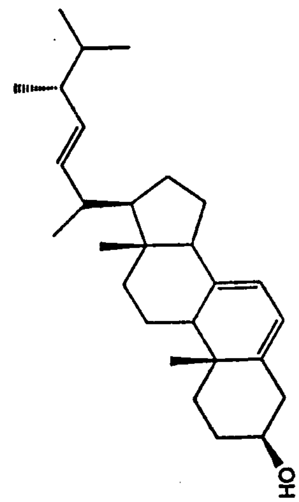

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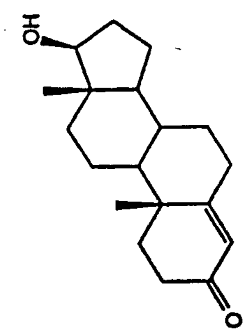
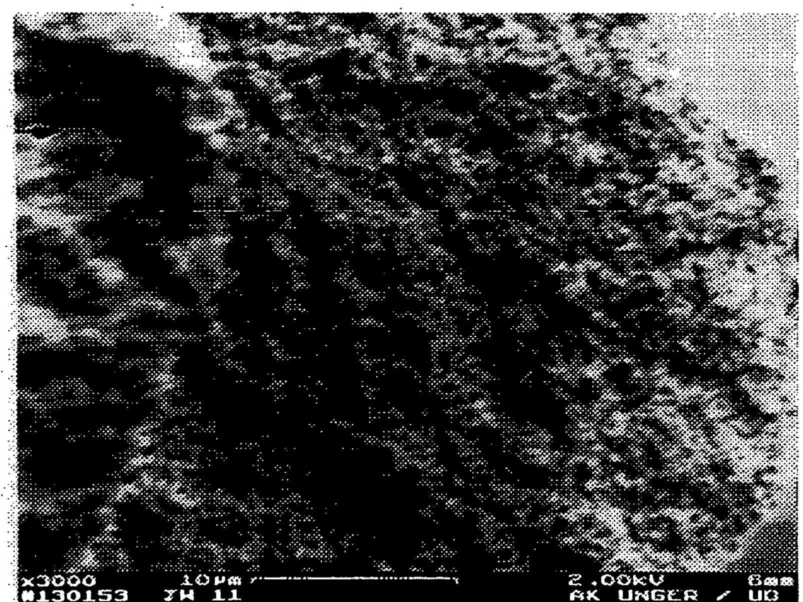


Tes

Fig. 1

a

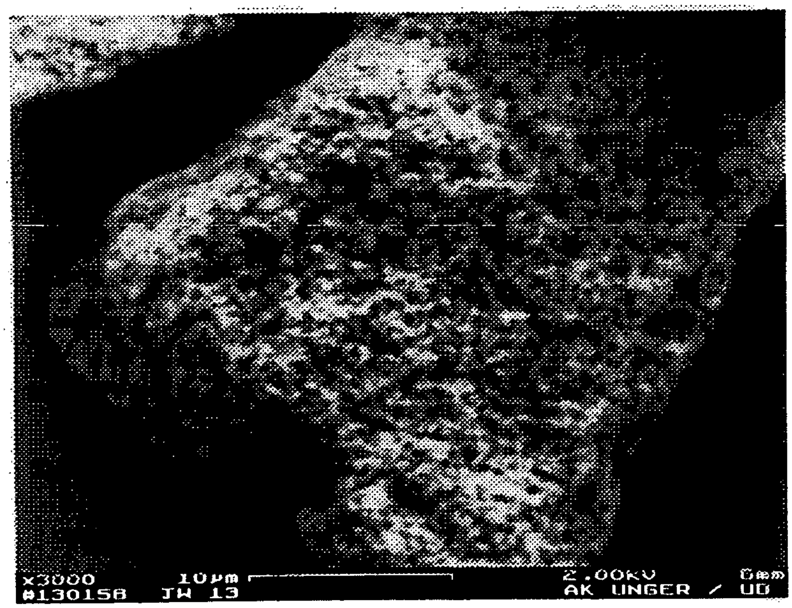

x3000 10µm
#130153 JW 11
2.00kV 6mm
AK UNGER / UD

b


x3000 10µm
#130155 JW 12
2.00kV 6mm
AK UNGER / UD

Fig. 1

c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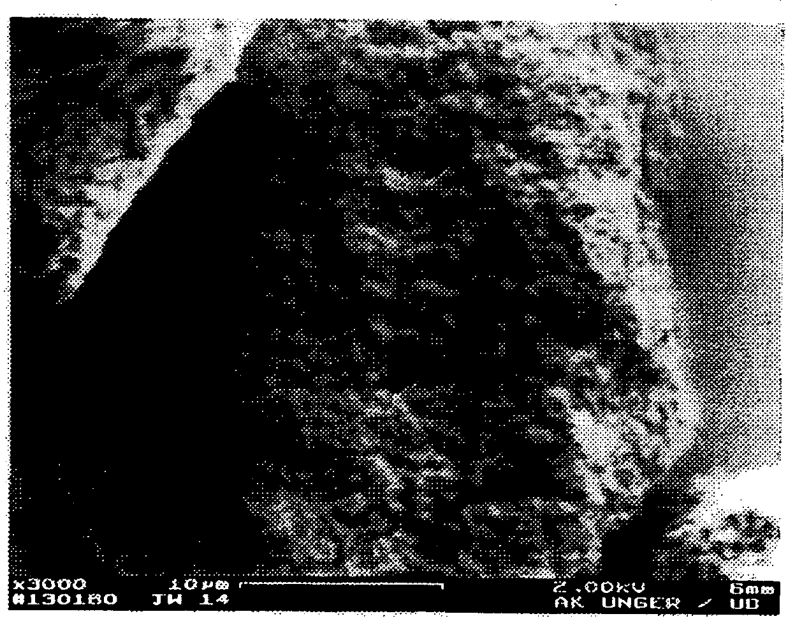
#130158 JW 13
2.00kV 6mm
AK UNGER / UD

d


x3000 10µm
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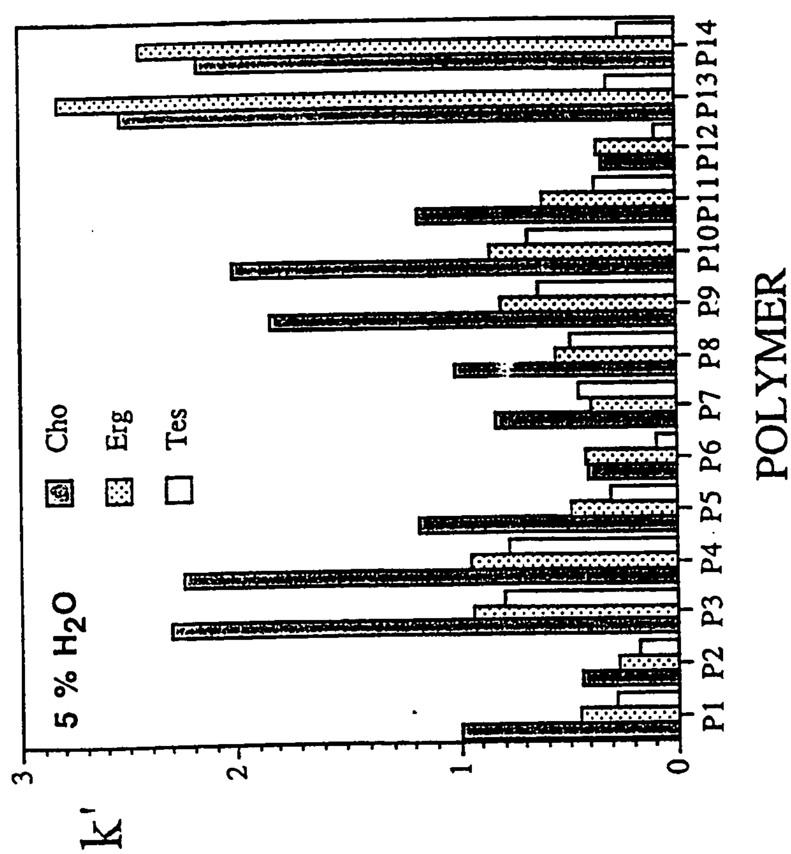
2.00kV 6mm
AK UNGER / UD

Figure 2A


k'
3
2
1
0
5 % H_2O
Cho
Erg
Tes
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
P12
P13
P14
POLYMER

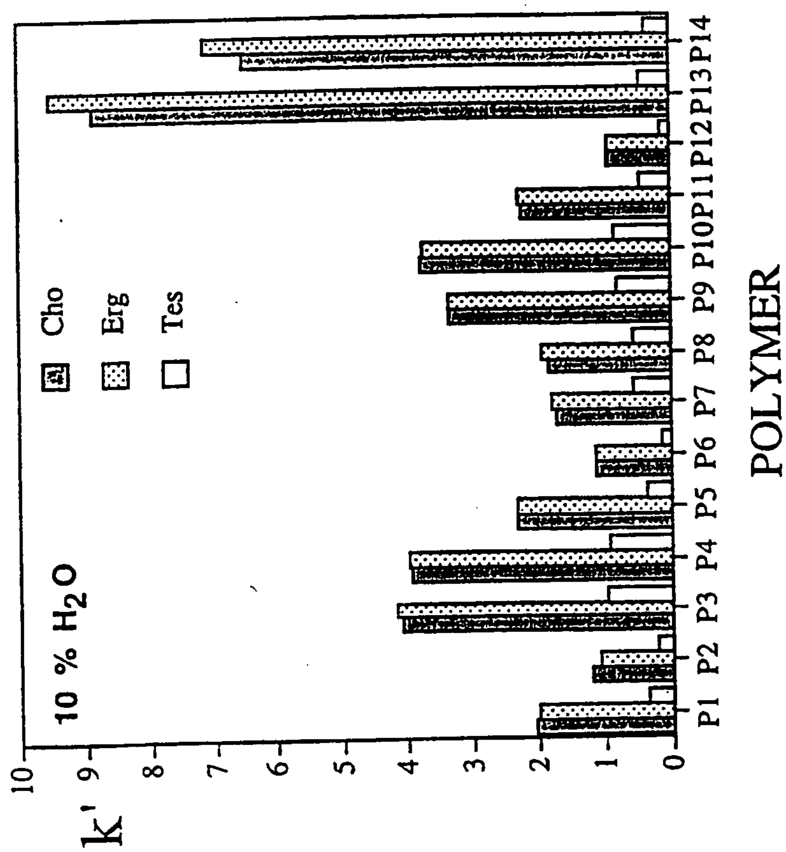

10 % H_2O
Cho
Erg
Tes
k'
0 1 2 3 4 5 6 7 8 9 10
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13 P14
POLYMER

Figure 2B

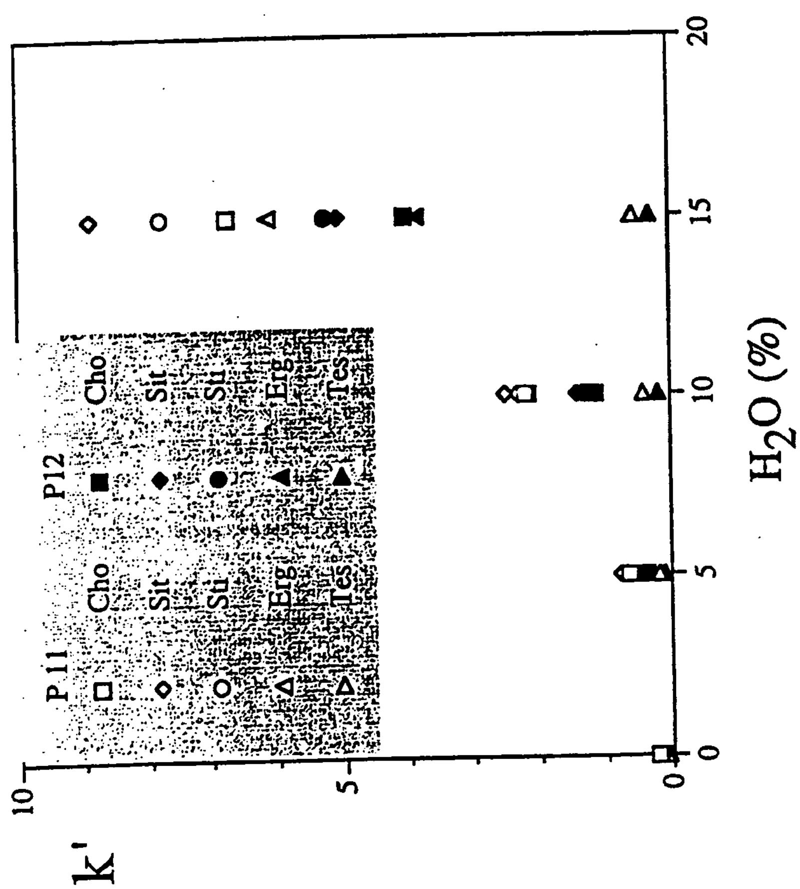
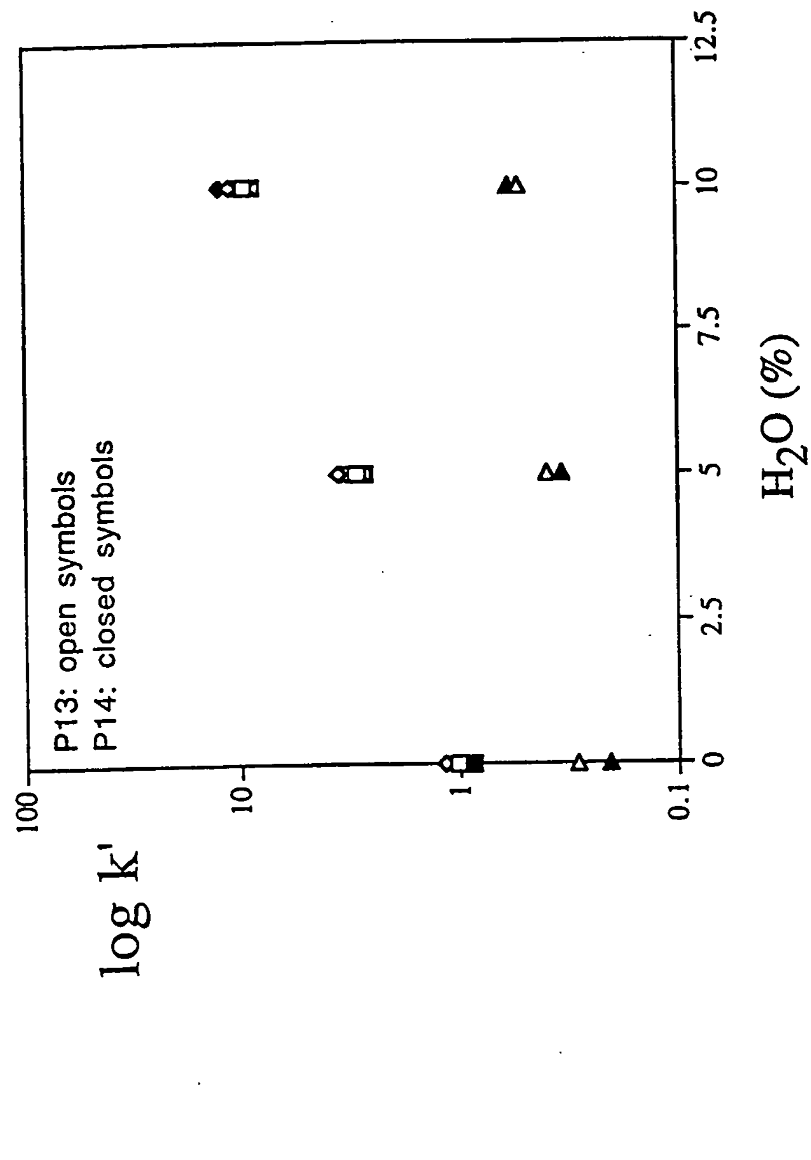

k'
10
5
0
0
5
10
15
20
H2O (%)
P 11
Cho
Sit
Sti
Erg
Tes
P12
Cho
Sit
Sti
Erg
Tes

Figure 3A


P13: open symbols
P14: closed symbols
log k'
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10
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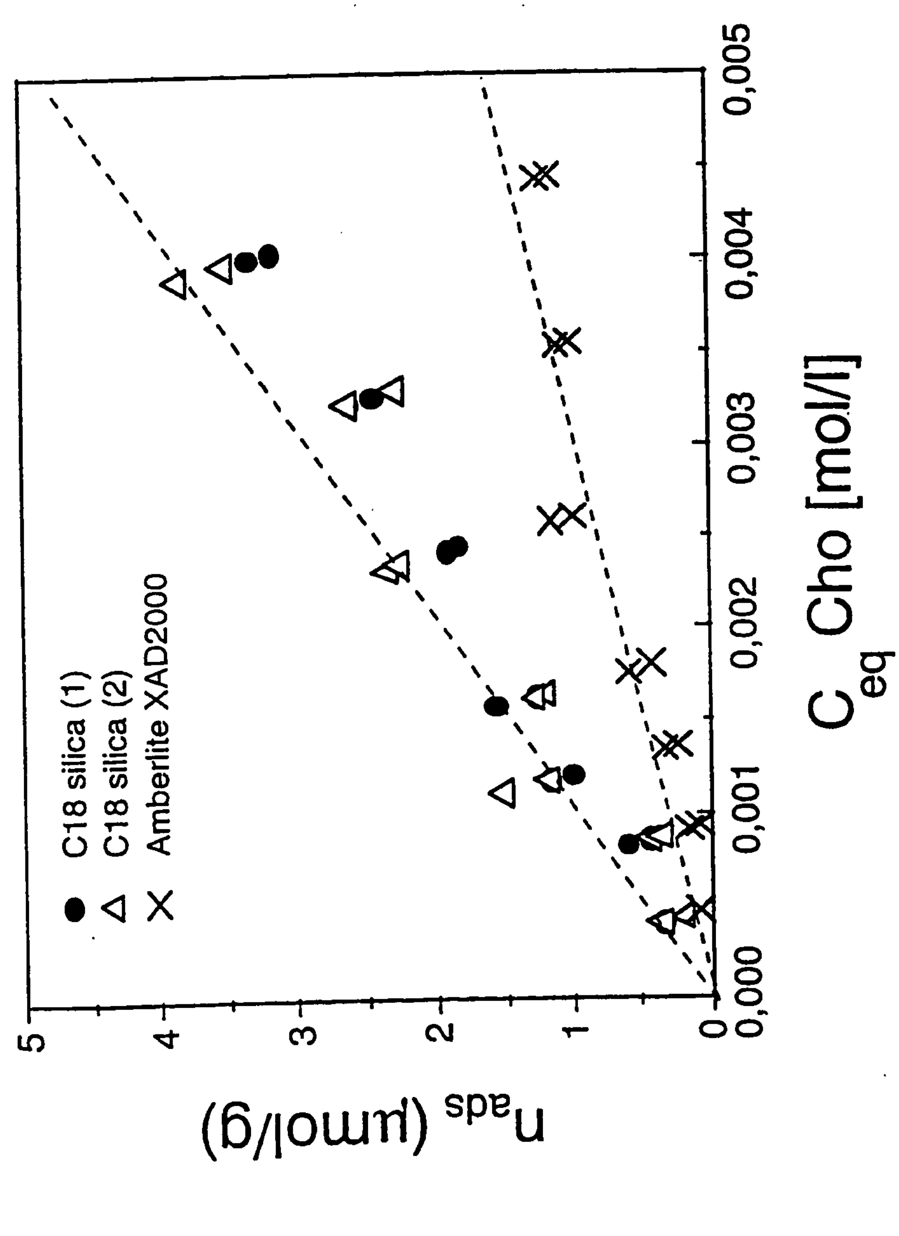
0
2.5
5
7.5
10
12.5
H_2O (%)

Figure 3B


C18 silica (1)
C18 silica (2)
Amberlite XAD2000
n_ads (µmol/g)
C_eq Cho [mol/l]
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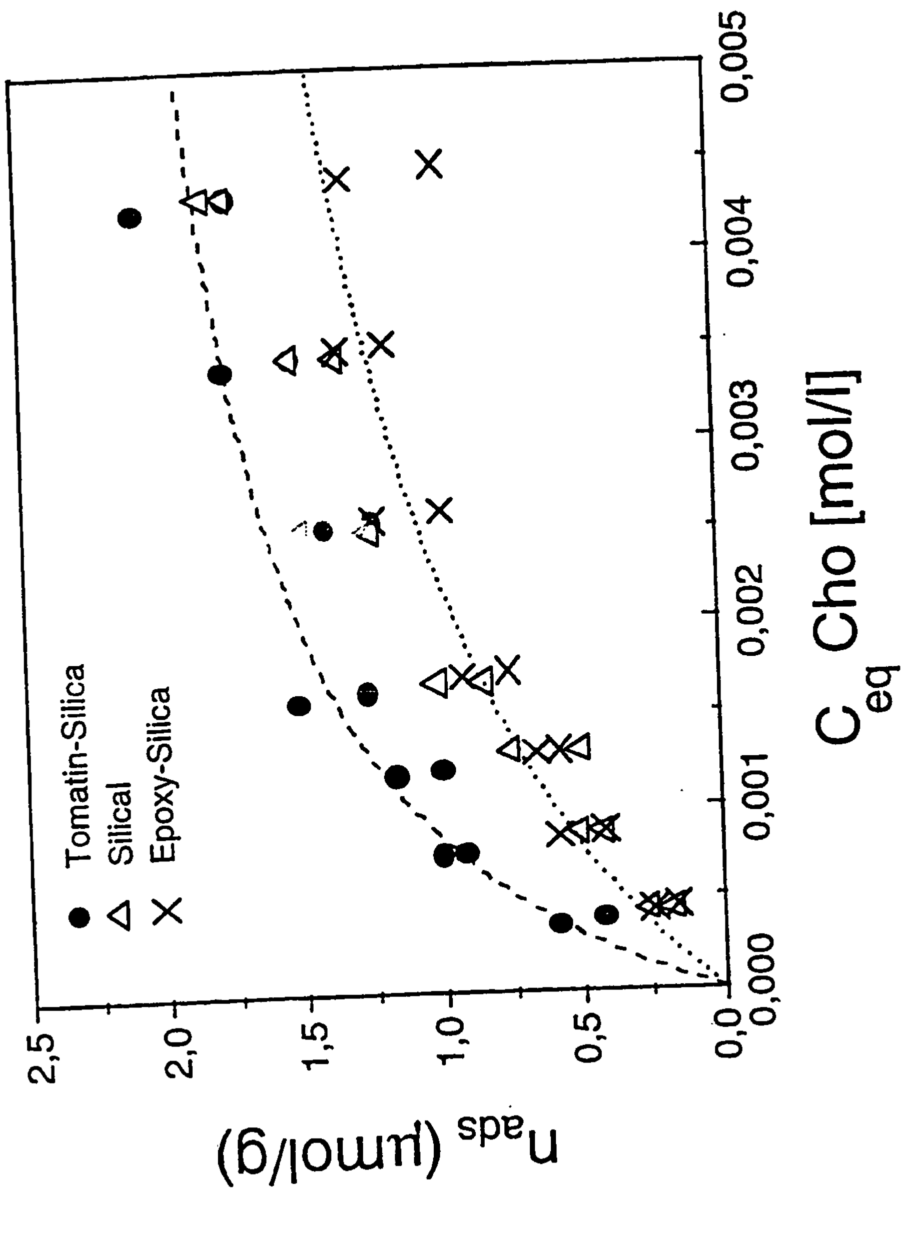
0,001
0,002
0,003
0,004
0,005
0
1
2
3
4
5

Figure 4A


Tomatin-Silica
Silical
Epoxy-Silica
n_{ads} (µmol/g)
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2,0
1,5
1,0
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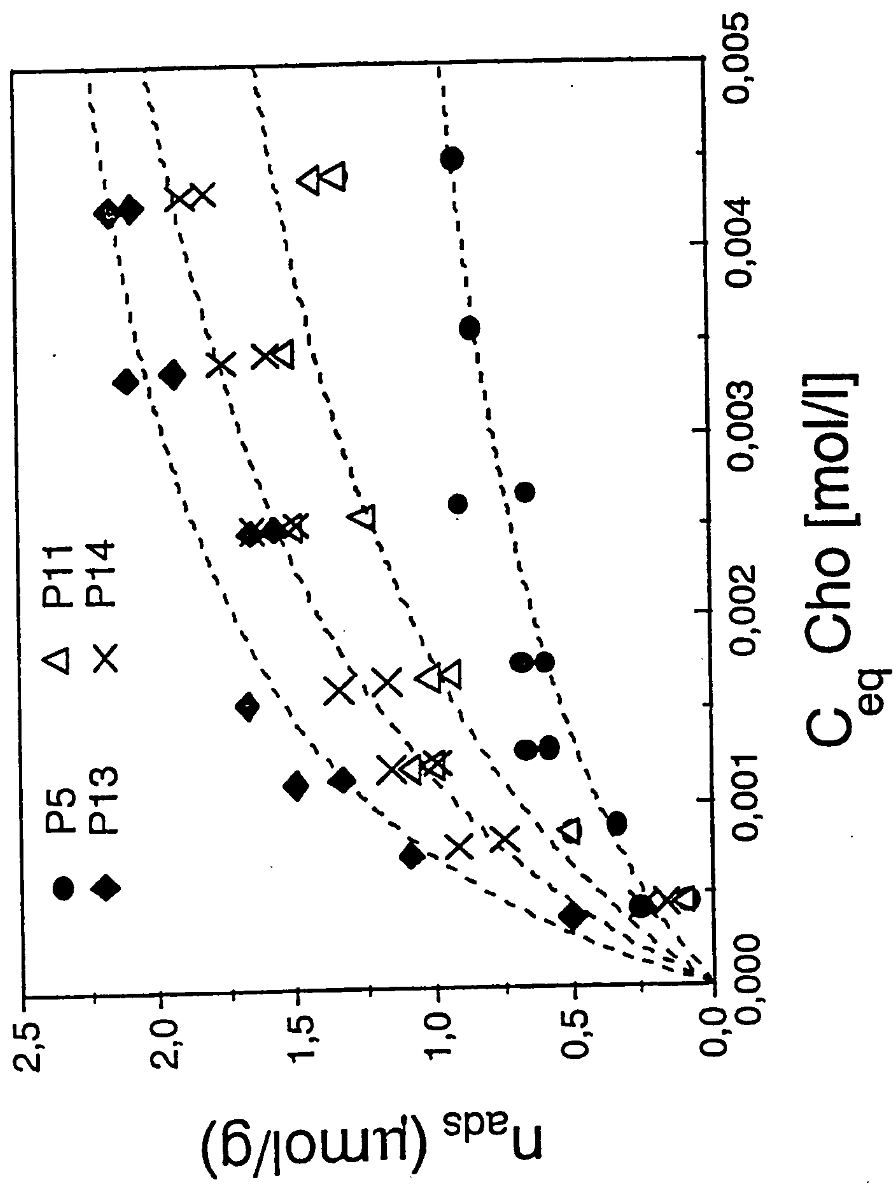
0,000
0,001
0,002
0,003
0,004
0,005
C_{eq} Cho [mol/l]

Figure 4B

Figure 4C


P5
P13
P11
P14
n_{ads} (µmol/g)
C_{eq} Cho [mol/l]
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0,5
1,0
1,5
2,0
2,5
0,000
0,001
0,002
0,003
0,004
0,005

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/03917

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48 A61K31/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SELLERGREN, BOERJE ET AL: "Imprinted Polymers for Selective Adsorption of Cholesterol from Gastrointestinal Fluids" CHEM. MATER. (1998), 10(12), 4037-4046 , December 1998 (1998-12), XP002116736 cited in the application the whole document ---	1-20
X	WHITCOMBE, MICHAEL J. ET AL: "A New Method for the Introduction of Recognition Site Functionality into Polymers Prepared by Molecular Imprinting: Synthesis and Characterization of Polymeric Receptors for Cholesterol" J. AM. CHEM. SOC. (1995), 117(27), 7105-11 , XP002116737 the whole document ---	14-16
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 September 1999	12/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Veronese, A

1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/03917

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	STYRBJORN E. B. ET AL: "Selective Reduction of Steroid 3- and 17-Ketones Using LiAlH4 Activated Template Polymers" J. AM. CHEM. SOC. (1993), 115(27), 2081-83 , XP002116738 See page 2082, column 2: " The rest of the imprint molecules are trapped within the polymer framework and inaccessible to chemical reactions" ---	1,4-8, 14-16
A	WO 97 38015 A (MOSBACH KLAUS ;RAMSTROEM OLOF (SE); IGEN INC (US)) 16 October 1997 (1997-10-16) the whole document ---	1-20
A	ASANUMA, HIROYUKI ET AL: "Molecularly imprinted polymer of.beta.-cyclodextrin for the efficient recognition of cholesterol" CHEM. COMMUN. (CAMBRIDGE) (1997), (20), 1971-1972 , XP002116739 cited in the application the whole document ---	1-20
A	K MOSBACH: "Molecular Imprinting" TIBS TRENDS IN BIOCHEMICAL SCIENCES, vol. 19, 1 January 1994 (1994-01-01), pages 9-14, XP002082394 ISSN: 0968-0004 the whole document -----	1-20

1

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 2-3
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 2-3 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 99/03917

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9738015 A	16-10-1997	AU 2721797 A	29-10-1997

Form PCT/ISA/210 (patent family annex) (July 1992)

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